The *Helicobacter pylori* Ferric Uptake Regulator (Fur) is Essential for Growth Under Sodium Chloride Stress

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Abstract

Epidemiological data and animal models indicate that *Helicobacter pylori* and dietary NaCl have a synergistic ill effect on gastric maladies. Here we show that the Ferric Uptake Regulator (Fur), which is a crucial regulatory factor required for *H. pylori* colonization, is essential for growth in the presence of high NaCl concentrations. Moreover, we demonstrate that the transcriptional response induced by sodium chloride stress exhibits similarities to that seen under iron depletion.

Helicobacter pylori can be found in the gastric mucosa of more than half of the human population (Pounder and Ng 1995). Long-term colonization results in gastritis and may lead to gastric or duodenal ulcers (Malaty 2007). Infection with *H. pylori* is also a risk factor for development of gastric cancer (Matysiak-Budnik and Megraud 2006; Mbulaiteye *et al*. 2009); to date, *H. pylori* is the only bacterium classified as a carcinogen (Anon. 1994). For colonized individuals, epidemiological data indicate a synergistic ill effect between diets rich in sodium chloride and *H. pylori* infection (Tsugane and Sasazuki 2007; Wang *et al*. 2009). The role of a high salt diet on *H. pylori* infection was also previously examined in several animal models. In a mouse model of infection, an increase in bacterial load was observed in animals consuming a high salt diet (Rogers *et al*. 2005). Whereas, in a gastric chemical carcinogenesis model of *H. pylori*-infected Mongolian gerbils, a high salt diet resulted in the dose-dependent promotion of cancer initiation that was accompanied by a shift in mucin production from the glandular to the surface mucous cells (Kato *et al*. 2006). Taken together, the epidemiological and animal based evidence suggest dietary mediated increases in the severity of *H. pylori*-induced disease outcomes.

Based on the above observations, we and others postulated and subsequently showed that salt concentration contributes to changes in expression of *H. pylori* virulence factors such as *cagA* (Loh *et al*. 2007) and *vacA* (Gancz *et al*. 2008) *in vitro*. However, the mechanisms of regulation of the salt stress response and the factors required for salt survival are not yet characterized for this organism.

In order to identify genes that contribute to *H. pylori's* ability to withstand salt stress, we took a targeted approach and screened defined *H. pylori* mutants that were constructed in the G27 strain background and already existed in our strain collection (Table 1). Specifically, we targeted strains containing mutations in known regulatory genes (*flgR, fur, luxS, spoT*) or known virulence genes (*cagA, ureA, vacA*). All *H. pylori* strains were maintained as frozen stocks at 80°C as previously described (Carpenter *et al*. 2010). Prior to each experiment, strains were revived on horse blood agar (HBA) plates as previously described (Carpenter *et al*. 2010) and used to inoculate liquid cultures that consisted of Brucella broth (Neogen

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Corporation) supplemented with 10% fetal bovine serum and 10µg/ml vancomycin (BB). These cultures were grown at 37°C with shaking at 100 rpm. Both liquid and plate cultures were grown under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) generated with an Anoxomat gas evacuation and replacement system (Spiral Biotech) in gas evacuation jars. When reviving mutant bacterial strains from the freezer only, plates were supplemented with 8µg/ml chloramphenicol (Cm) (EMD Chemicals, Inc.) and/or 25µg/ml kanamycin (Kan) (Gibco) where appropriate (Table 1).

To determine whether mutation of any of the *H. pylori* regulatory or virulence genes affected growth in the presence of elevated salt concentrations, we calculated the plating efficiency of each mutant strain and the wildtype G27 control strain by determining the number of colonies obtained after spotting on solidified (1.3% agar final) BB supplemented with various concentrations of NaCl. The normal solidified BB medium contains 5 gr/liter of NaCl (BB5), and we achieved increased salt concentration by use of BB containing 13.5 gr/ liter of NaCl (BB13.5). This elevated concentration of sodium chloride is lower than the dramatic growth inhibitory condition we previously described for this strain (BB15) (Gancz *et al*. 2008). The wildtype strain and each of the defined isogenic mutant strains were grown to mid-exponential phase in liquid media and then O.D. equilibrated to give identical cell densities. Cells were then serially diluted and plated side-by-side on BB5 and BB13.5 plates (Figure 1) as well as on HBA plates (data not shown).

Comparison of the plating data revealed that while wildtype G27 showed no decrease in plating efficiency on BB13.5, the resulting colonies were significantly smaller than on non-supplemented media (Figure 1). This fact suggests that the concentration of NaCl found in the BB13.5 plates was sufficient to create stressful conditions under which the bacteria can grow but at a slower rate. Disruption of *cagA, flgR, luxS, ureB* or *vacA* all resulted in plating efficiencies that were similar to those of the wildtype (data not shown). However, disruption of the ferric uptake regulator, *fur*, or *spoT* resulted in a significant reduction in the plating efficiency of these mutant strains on high salt media (Figure 1 panel A and data not shown). Given that the *fur* mutant strain showed the most drastic growth defect at elevated salt concentrations and because Fur is known to regulate additional stress responses besides the response to iron ion concentration (Bijlsma *et al*. 2002; Carpenter *et al*. 2010), has unique features in *H. pylori* gene regulation (Delany *et al*. 2001; Ernst *et al*. 2005; Miles *et al*. 2010a) and is the scope of on-going research in our laboratory (Carpenter *et al*. 2007; Carpenter *et al*. 2009; Carpenter *et al*. 2010; Gancz *et al*. 2006; Miles *et al*. 2010a; Miles *et al*. 2010b), we chose to further investigate the role of *fur* in the response to salt stress.

First, to conclusively demonstrate that the *fur* mutation was responsible for the demonstrated salt sensitive phenotype, we attempted to complement the sensitivity phenotype using pDSM340, which is a plasmid that carries a wildtype copy of *fur* (Carpenter *et al*. 2007). Transformation of this plasmid into the Δ*fur* background resulted in complete restoration of growth to the wildtype phenotype (Figure 1, panel A). Therefore, we conclude that *fur* is essential for growth under these conditions.

Next, given that *H. pylori* is considered a panmictic species (Salaun *et al*. 1998) and since increasing evidence indicates the occurrence of strain specific phenomenon (Gancz *et al*. 2008), we next investigated the necessity of *fur* for growth under high salt conditions in two additional strain backgrounds. To this end, we compared the plating efficiency of strains HP26695 and HP43504 to that of their isogenic Δ*fur* mutants, which were constructed as previously described (Carpenter *et al*. 2009; Gancz *et al*. 2006). We noticed that although the various wildtype strains differed in their overall salt sensitivity, each of the Δ*fur* strains showed a growth defect in comparison to their respective parental strain (Figure 1, panel B). This indicates that the requirement of Fur for growth under salt stress conditions is not strain

dependent. Similar to G27, the other *H. pylori* strains also exhibited smaller colonies on BB13.5, indicating that they were experiencing stressful conditions (Figure 2, panel B).

Fur is known to be an important regulatory factor in *H. pylori* that has been shown to control the expression of a large number of genes (Danielli *et al*. 2006; Ernst *et al*. 2005; Gancz *et al*. 2006; Lee *et al*. 2004). Included among these genes is *amiE*, which encodes an aliphatic amidase that is regulated by the iron-bound form of Fur (van Vliet *et al*. 2003), and *pfr*, which encodes a nonheme iron-containing ferritin that is regulated by the *apo* form of Fur (Bereswill *et al*. 2000). Thus, to determine if Fur regulated processes were taking place during salt stress in *H. pylori*, we monitored the relative transcription of these two known Fur dependent genes by RNAse protection assay (RPA) as previously described (Carpenter *et al*. 2007). Additionally, we monitored the Fur-dependent expression of *vacA* and *cagA*, two virulence genes whose salt-dependent transcription we previously examined (Gancz *et al*. 2008). RPAs were conducted with RNA harvested from cultures of the wildtype strain and the isogenic *fur* mutant strain that were grown in BB5 and then exposed to a salt shock for one hour. To accomplish this, overnight cultures of each strain were harvested by centrifugation and then exposed to salt shock conditions by resuspension of the cells in BB containing 25 gr/liter of NaCl (BB25). RNA was extracted from bacterial cells, the *amiE, pfr, cagA* and *vacA* riboprobe templates were generated by PCR, radiolabeled riboprobes were produced, and the RPAs were performed all as previously described (Carpenter *et al*. 2010; Gancz *et al*. 2008). The gels were subsequently exposed to phosphor screens, which were scanned using a FLA-5100 scanner (FujiFilm). Data were then analyzed/quantitated using the Multi-Gauge software (version 3.0, FujiFilm).

Examination of the response of the virulence factors *vacA* and *cagA* to salt shock by RPAs demonstrated that, as expected for strain G27 (Gancz *et al*. 2008), upon exposure to increased salt, *vacA* expression was increased by 10.55 fold, while *cagA* expression was virtually unchanged (Figure 2). These responses were almost identical in the *fur* mutant strain (Figure 2), indicating that Fur does not function as a critical regulatory factor for *cagA* and *vacA* in these conditions. Conversely, Fur does function as a critical regulator of *amiE* and *pfr* in these conditions; in the wildtype strain, *amiE* expression was increased upon salt shock, while *pfr* expression was decreased, but these changes were completely lost in the *fur* mutant strain (Figure 2). Interestingly, these changes in expression closely resemble the previously characterised transcriptional response of these genes to iron depletion (Carpenter *et al*. 2007; Carpenter *et al*. 2009). For example, *amiE* expression in response to salt stress increased 2.03 fold in comparison to 5.8 fold under iron depletion (Carpenter *et al*. 2007), while the *pfr* transcript showed a 5.6 decrease under salt shock conditions and a 3.3 fold decrease under iron depletion (Carpenter *et al*. 2007). Taken together, these data suggest that for *H. pylori*, salt shock mimics iron depletion and that Fur plays a crucial role in mediating changes in gene expression that allow growth under these conditions.

The involvement of Fur in the response to high concentrations of salt was previously also suggested for other microorganisms: *Desulfovibrio vulgaris* (Bender *et al*. 2007; He *et al*. 2010; Mukhopadhyay *et al*. 2006), the halophilic bacterium *Chromohalobacter salexigens* (Argandona *et al*. 2010), *Bacillus subtilis* (Hahne *et al*. 2010; Hoffmann *et al*. 2002; Steil *et al*. 2003), and *Bacillus cereus* (den Besten *et al*. 2009). For *D. vulgaris*, microarray analysis demonstrated that many of the genes showing marked changes in transcription following salt exposure belonged to the Fur regulon. Despite this, the wildtype *D. vulgaris* and the *Δfur* strain display virtually identical suppressed growth curves under elevated salt conditions. However, the *fur* mutant strain differs from the wildtype in that while growth of the wildtype in salt can be restored by the addition of glycine betaine as an osmoprotectant, the *fur* mutant strain is unable to be rescued from salt induced poor growth (Bender *et al*. 2007). In *B. subtilis* the connection between Fur and salt stress was inferred from proteomic

examination of the response to high salt conditions (Hoffmann *et al*. 2002) and was further studied by comparative transcriptional profiling in response to iron depletion and salt shock (Hahne *et al*. 2010; Steil *et al*. 2003). Interestingly, in this organism addition of iron to the growth media partially reversed the *B. subtilis* growth defect induced by salt-stress (Hoffmann *et al*. 2002). Similarly, in the closely related bacterium, *B. cereus*, salt exposure causes induction of genes involved in iron homeostasis (den Besten *et al*. 2009). Thus, while it is clear that in *H. pylori* Fur acts as a global transcriptional regulator that responds to and or mediates the response to an assortment of environmental signals (Bijlsma *et al*. 2002; Carpenter *et al*. 2010), we suggest that the link between salt, iron and mediation of the osmotic stress response by Fur crosses bacterial taxa and may prove to be a general one.

Acknowledgments

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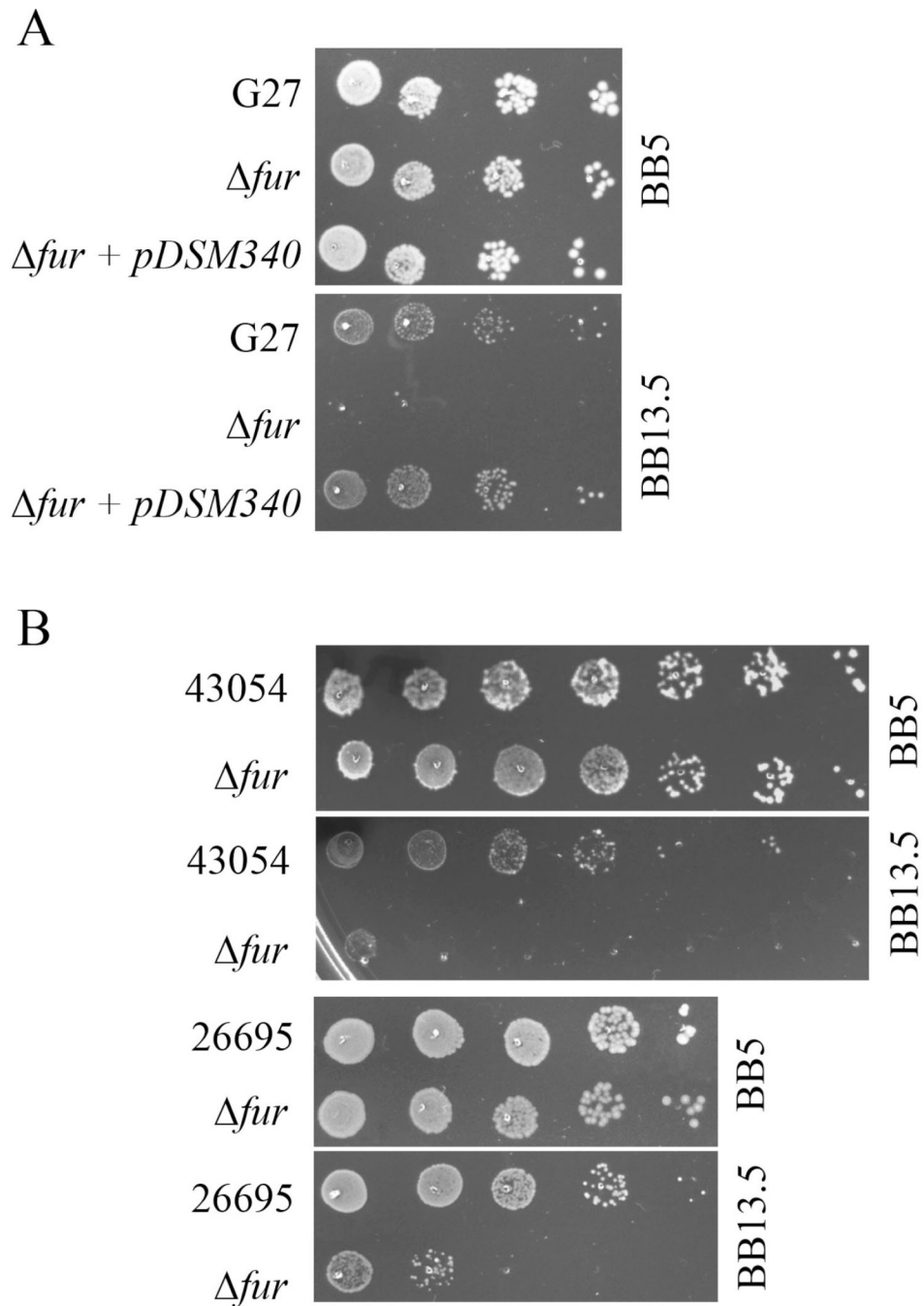
Figure 1.
Growth characteristics of *H. pylori* and isogenic Δ*fur* mutants on normal (BB5) and elevated salt (BB13.5) solidified media. **A.** Relative growth of G27 wildtype, the isogenic Δ*fur* mutant (DSM300), and the *fur* complemented strain (DSM343) on BB5 and BB13.5 plates are shown. Ten-fold dilutions of the indicated strain were spotted on solidified Brucella broth based medium containing 5 gr/liter NaCl (BB5) (upper panel) or 13.5 gr/liter NaCl (BB13.5) (lower panel). **B.** Relative growth of strains 43054 and 26695 and their isogenic Δ*fur* mutant strains on BB5 and BB13.5 plates are shown. Ten-fold dilutions of the indicated strain were spotted on solidified Brucella broth based medium containing 5 gr/liter NaCl

(BB5) (upper panel) or 13.5 gr/liter NaCl (BB13.5) (lower panel). The results shown are representative of multiple biological repeats.
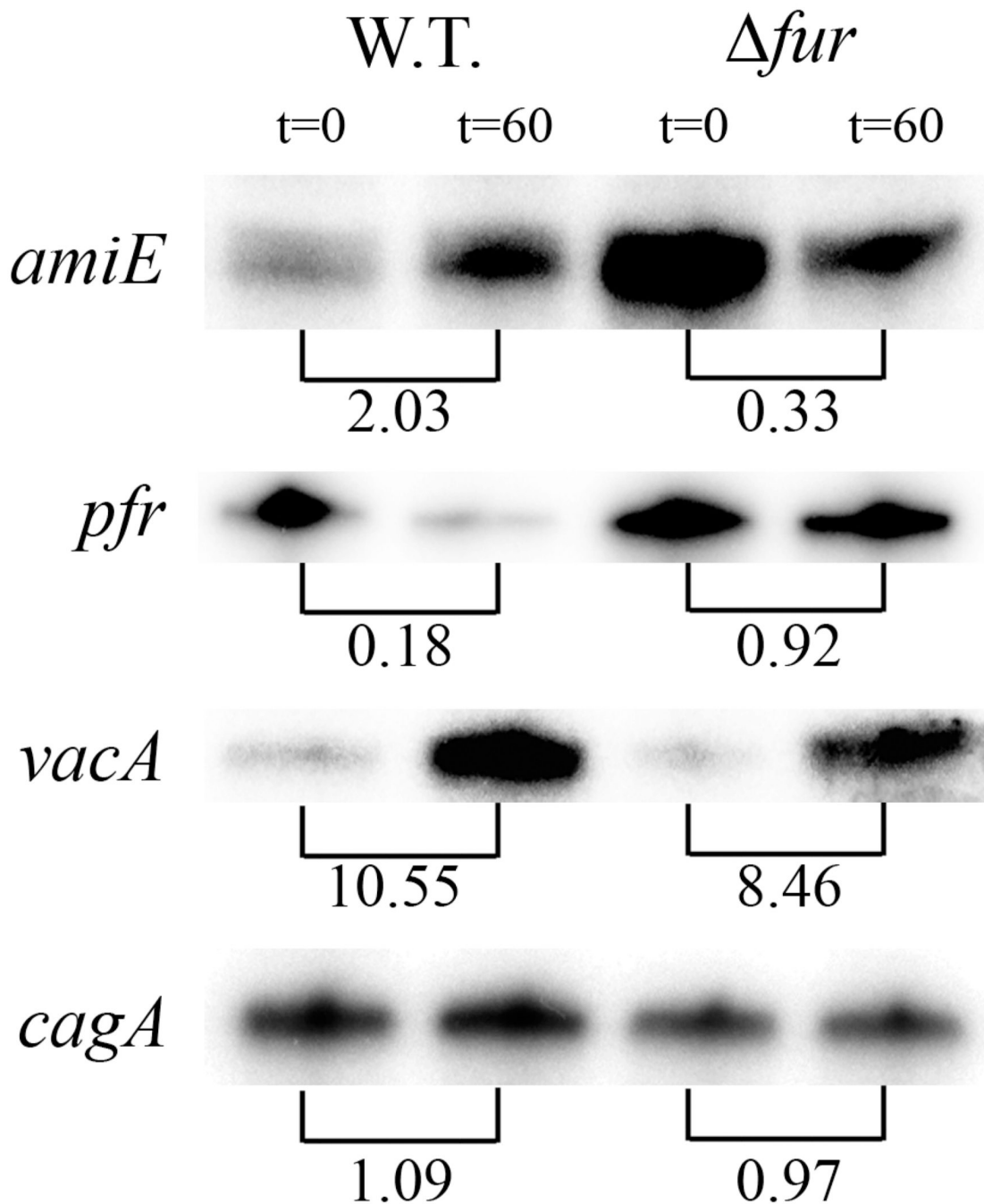
Figure 2.
Transcription of *amiE, pfr, vacA* and *cagA* following salt shock as assessed by RNAse protection assay (RPA). Logarithmically growing liquid cultures of G27 and the isogenic Δ*fur* mutant strain were salt shocked by replacing the BB5 growth medium with BB25 medium (containing 25 gr/liter NaCl). Samples were taken immediately after the cells were resuspended in BB25 (t=0) and one hour after the change of the medium (t=60). RNA was harvested, and RPAs were conducted as described in the text. Protected bands for the indicated transcript are shown for each strain. The fold change in expression from the t=0 time point is indicated. These data are representative of three independent biological experiments.

TABLE 1

Plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pDSM142	*pΔ*HP1027-K7	(Gancz *et al.* 2006)
pDSM340	*fur*, pTM117 complementation vector	(Carpenter *et al.* 2007)
H. *pylori* strains		
G27	WT *H. pylori*	(Covacci *et al.* 1993)
26695	WT *H. pylori*	(Tomb *et al.* 1997)
43504	WT *H. pylori*	(Alamuri *et al.* 2006)
DSM43	G27 *ureB*::*aphA*, Kan^r	(Joyce *et al.* 2001)
DSM90	G27 *flgR*::*cat*, Cm^r	This study
DSM205	G27 *vacA*::*aphA*, Kan^r	(Amieva *et al.* 2002)
DSM207	G27 *cagA*::*cat*, Cm^r	(Amieva *et al.* 2002)
DSM211	G27 *luxS*::*aphA*, Kan^r	Kind gift from J.V. Solnick laboratory
DSM300	G27 Δ*fur*::*cat*, Cm^r	(Carpenter *et al.* 2007)
DSM343	DSM300 (pDSM340), Kan^r Cm^r	(Carpenter *et al.* 2007)
DSM357	26695 Δ*fur*::*cat*, Cm^r	(Carpenter *et al.* 2009)
DSM388	43504 Δ*fur*::*aphA*, Kan^r	This study
DSM594	G27 *spoT*::*aphA*, Kan^r	(Zhou *et al.* 2008)